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Gamma-Irradiation Reduces the Allogenicity of Donor Corneas

William Stevenson, Sheng-Fu Cheng, Parisa Emami-Naeini, Jing Hua, Eleftherios I. Paschalis, Reza Dana, and Daniel R. Saban*

PURPOSE. To evaluate the utility and allogenicity of gamma-irradiated corneal allografts.

METHODS. Corneal buttons were harvested from C57BL/6 mice and decellularized with gamma irradiation. Cell viability was assessed using TUNEL and viability/cytotoxicity assays. Orthotopic penetrating keratoplasty was performed using irradiated or nonirradiated (freshly excised) C57BL/6 donor grafts and BALB/c or C57BL/6 recipients. Graft opacity was assessed over an 8-week period and graft survival was evaluated using Kaplan-Meier survival curves. Mixed-lymphocyte reactions and delayed-type hypersensitivity assays were performed to evaluate T-cell alloreactivity. Real-time PCR was used to investigate the corneal expression of potentially pathogenic T-helper 1, 2, and 17 cell-associated cytokines.

RESULTS. Corneal cells were devitalized by gamma irradiation as evidenced by widespread cellular apoptosis and plasma membrane disruption. Nonirradiated allograft and isograft rates of survival were superior to irradiated allograft and isograft rates of survival ($P < 0.001$). Mixed lymphocyte reactions demonstrated that T-cells from irradiated allograft recipients did not exhibit a secondary alloimmune response ($P < 0.001$). Delayed-type hypersensitivity assays demonstrated that irradiated allografts did not elicit an alloreactive delayed-type hypersensitivity response in graft recipients ($P \leq 0.01$). The corneal expression of T-helper 1, 2, and 17 cell-associated cytokines was significantly lower in failed irradiated allografts than rejected nonirradiated allografts ($P \leq 0.001$).

CONCLUSIONS. Gamma-irradiated corneas failed to remain optically clear following murine penetrating keratoplasty; however, gamma irradiation reduced the allogenicity of these corneas, potentially supporting their use in procedures such as anterior lamellar keratoplasty or keratoprosthesis implantation. (*Invest Ophthalmol Vis Sci.* 2012;53:7151-7158) DOI: 10.1167/iovs.12-9609

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Corneal transplantation, also known as corneal grafting, is one of the oldest, most common, and most successful forms of solid tissue transplantation. In 2010, over 42,000 corneal transplantations were performed in the United States alone.¹ Despite advances in recognition and treatment, immune rejection remains the leading cause of corneal transplantation failure.² Corneal allografts placed in first-time, noninflamed, avascular graft beds enjoy a 2-year survival rate approaching 90%; however, previously sensitized, inflamed, or vascularized graft beds afford a much lower rate of graft acceptance.^{3,4} Although corneal allograft rejection can be mediated by redundant immunologic processes, currently available evidence indicates that T-cells, particularly CD4⁺ T-cells, have a critical role in the immunopathogenesis of corneal allograft rejection.^{5,6} Treatment modalities that downregulate CD4⁺ T-cell alloreactivity promise to enhance the long-term survival of corneal allografts.

According to the World Health Organization, corneal blindness accounts for nearly 8 million of the 39 million cases of blindness worldwide.^{7,8} Corneal transplantation is a sight-saving procedure with the potential to benefit many of those who suffer from corneal blindness; unfortunately, it has been estimated that only 100,000 corneal transplantations are performed per year worldwide.⁹ The availability of corneal transplantation is limited in part by the shortage of donated corneas, exclusion of corneas not fit for transplantation, and relatively short shelf-life of transplantation-suitable corneas.¹⁰ Novel corneal processing methods, such as gamma irradiation, could help address the worldwide shortage of corneal tissue by increasing the supply and extending the shelf-life of corneas available for transplantation.

Gamma irradiation is commonly used by tissue banks for the sterilization of grafts against bacterial, viral, fungal, and even prional contaminants.¹¹⁻¹³ TBI/Tissue Banks International (Baltimore, MD) uses specialized procedures for the procurement, screening, gamma irradiation, and preservation of donor corneas to produce VisionGraft Sterile Cornea.¹⁴ Gamma-irradiated corneas have been used in a variety of clinical procedures, including anterior lamellar keratoplasty, tectonic keratoplasty, glaucoma patch grafting, and keratoprosthesis implantation.^{15,16} Thus far, irradiated corneas have only been used for procedures that do not require viable graft endothelium. We evaluated the utility of gamma-irradiated corneal grafts using a murine model of orthotopic penetrating keratoplasty. Furthermore, since gamma irradiation has been shown to reduce immunogenicity in some experimental models, we investigated the allogenicity of gamma-irradiated corneas.

MATERIALS AND METHODS

Animals and Anesthesia

Male C57BL/6 and BALB/c mice (Charles River Laboratories, Wilmington, MA) aged 8 to 10 weeks were used for this study. Mice were

housed in a secure, pathogen-free environment at the Schepens Eye Research Institute Animal Care Facility. All procedures and protocols were approved by the Schepens Eye Research Institute Animal Care and Use Committee, and all animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research. Anesthesia and analgesia were induced with intraperitoneal injections of ketamine/xylazine at doses of 120 and 20 mg/kg, respectively.

Tissue Harvesting and Gamma Irradiation

The center of each donor cornea (C57BL/6) was marked with a 2 mm diameter trephine and excised using Vannas scissors (Storz Instruments Co., Madison, NJ). Following excision, corneas were placed in chilled Optisol-GS (Bausch & Lomb, Irvine, CA) and shipped to TBI/Tissue Banks International for gamma irradiation.¹¹ Nonirradiated (freshly excised) corneal grafts (C57BL/6) were harvested, placed in chilled PBS, and used immediately for corneal transplantation.

For the TUNEL assay, freshly enucleated eyes were placed in chilled Optisol-GS (Bausch & Lomb). Gamma irradiation was performed using a synthetic radioactive isotope of Cobalt-60 at the Massachusetts Institute of Technology Radiation Lab. The dose of gamma irradiation administered was calculated using the standard decay curve of Co-60. Naïve and gamma-irradiated eyes were stored at +4°C until further processing.

TUNEL Assay

TUNEL was performed using a commercially available kit as described previously (in situ cell death detection kit; Roche Diagnostics Corp., Indianapolis, IN).¹⁷ In brief, naïve and gamma-irradiated eyes were embedded in optimal cutting temperature (OCT) compound and frozen. Cryosection was performed and 13 µm sections were placed on positively charged glass slides (Menzel Gläser, Braunschweig, Germany). The sections were fixed in 4% paraformaldehyde for 20 minutes and washed in PBS for 30 minutes at room temperature. Samples were permeabilized on ice for 2 minutes in a solution containing 0.1% Triton X-100 and 0.1% sodium citrate. Four groups were examined: gamma-irradiated eyes (experimental group), nonirradiated eyes (control negative), nonirradiated eyes treated with DNase (control positive), and gamma-irradiated eyes stained with label solution alone (control false positive). TUNEL and label solutions were prepared as recommended, and the treated samples were incubated in a humidified chamber at 37°C for 60 minutes in the dark. Samples were washed thoroughly in PBS and mounted using mounting media with 4',6-diamidino-2-phenylindole (DAPI). The stained corneal sections were examined using a confocal microscope (Leica TCS-SP5; Leica Microsystems, Buffalo Grove, IL) at 40× magnification.

Cell Viability/Cytotoxicity Assay

Cell viability was assessed with a commercially available kit as described previously with minor modifications (LIVE/DEAD viability/cytotoxicity kit for mammalian cells; Invitrogen, Carlsbad, CA).¹⁸ Freshly excised naïve corneas and gamma-irradiated corneas were washed 3 times in sterile PBS for 10 minutes. Immediately before staining, a solution containing 2.5 µL calcein AM (4 mM in dimethyl sulfoxide [DMSO]) and 10 µL ethidium homodimer-1 (2 mM in 1:4 DMSO/H₂O) in 5 mL PBS was prepared. Corneal buttons were incubated in this solution for 40 minutes in the dark at room temperature. Corneas subsequently were washed one time in PBS for 5 minutes and mounted using mounting media with DAPI. Samples were examined using a confocal microscope (Leica TCS-SP5; Leica Microsystems) at 40× magnification.

Corneal Transplantation

Murine orthotopic penetrating keratoplasty was performed as described previously using irradiated corneas and nonirradiated

corneas as donor grafts.¹⁹ Briefly, the center of each recipient's right eye was marked with a 1.5 mm diameter trephine and excised. The donor corneal graft was centered on the graft bed and secured using 8 interrupted 11-0 nylon sutures (Surgical Specialties Co., Reading, PA). Tarsorrhaphies were placed for the first 2 days post-transplantation and corneal sutures were removed on postoperative day 7. Corneal transplantations that experienced complications (e.g., cataract, infection, anterior synechiae, or intraoperative hemorrhage) were excluded from analysis.

Immunosuppressive Regimen

Dexamethasone was prepared by dissolving powdered dexamethasone (Sigma-Aldrich, St. Louis, MO) in a mixture of PBS and DMSO (Calbiochem, Darmstadt, Germany) at a concentration of 1.0%. Immunosuppressed mice received either topical or combined topical and systemic dexamethasone using previously established doses.²⁰ Dexamethasone 0.1% eye drops were administered to transplanted eyes twice per day from days 0 to 14. Intraperitoneal dexamethasone (2 mg/kg) was administered once per day from preoperative day 1 to postoperative day 5.

Evaluation of Graft Opacity and Survival

Slit-lamp biomicroscopy was performed to evaluate graft opacity over an 8-week period. A standard opacity-grading system was used to score opacity in a semiquantitative manner, with each graft receiving a score from 0 to 5⁺ based on the presence and extent of opacity as described previously.¹⁹ Graft failure was defined as two consecutive time points with a score of 3⁺, denoting obscuration of the iris details. Kaplan-Meier survival curves were used to analyze graft survival.

Mixed Lymphocyte Reaction (MLR)

The MLR protocol used has been described previously.²¹ Briefly, transplant recipient's ipsilateral submandibular and cervical lymph nodes were harvested and pooled during the peak of allosensitization that occurs around post-transplantation week 3. T-cells were sorted magnetically using anti-CD90.2 magnetic microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's recommendations. The spleens of naïve C57BL/6 mice were harvested and erythrocytes were lysed to obtain unfractionated stimulator cells that subsequently were incubated for 20 minutes at 37°C in the presence of 50 µg/mL mitomycin-C (Sigma-Aldrich). T-cells and stimulator cells were co-cultured at a 1:1 ratio in a 96-well round bottom plate for 72 hours at 5.0% CO₂. Mixed lymphocyte reaction co-cultures were pulsed with BrdU (Sigma-Aldrich) 16 hours before the measurement of T-cell proliferation with a BrdU incorporation assay kit (Millipore, Billerica, MA) according to the manufacturer's recommendations.

Delayed-Type Hypersensitivity (DTH) Assay

The DTH assay used has been described previously.²² In brief, naïve C57BL/6 spleens were harvested, erythrocytes were lysed, and unfractionated splenocytes were diluted to a concentration of 1.5×10^6 cells per 10 µL. The ipsilateral ear pinna of each transplant recipient was injected with 10 µL of the stimulator cell suspension. Ear thickness was measured using an engineer's micrometer (Mitutoyo, Aurora, IL) at 3 time-points: pre-injection, and 24 and 48 hours post-injection. At each time point, three measurements were taken and averaged. Specific ear swelling (Δ ear thickness) was calculated by subtracting average baseline measurements from the peak average maximal ear thickness measured at either 24 or 48 hours post-injection.

Real-Time PCR

Recipient corneas from mice sacrificed for the MLR were harvested and pooled randomly within their respective groups. Total RNA was

extracted using Trizol Reagent (Invitrogen) and the commercially available RNeasy Microkit (Qiagen, Valencia, CA). The first strand of complementary DNA was synthesized using random hexamers (SuperScript III; Invitrogen) according to the manufacturer's recommendations. Real-time PCR was performed using TaqMan Universal PCR Mastermix and dye-labeled, predesigned primers (Applied Biosystems, Carlsbad, CA) for IFN- γ (Mm01168134_m1), IL-17A (Mm00439618_m1), IL-4 (Mm00445259_m1), and GAPDH (Mm99999915_g1), and all assays were performed in triplicate. Results were derived by the comparative threshold cycle method and normalized to GAPDH as the internal control (copy number/ 10^6 GAPDH).

Statistical Analysis

Data were analyzed using ANOVA and Dunnett's multiple comparison test or Student's *t*-test. Kaplan-Meier survival curves were analyzed using the log-rank (Mantel-Cox) test. Results are presented as the mean \pm 95% confidence interval (CI), and error bars represent the 95% CI. *P* values \leq 0.05 were considered statistically significant.

RESULTS

Corneal Cells Were Devitalized by Gamma Irradiation

To evaluate corneal cell viability, gamma-irradiated corneas were compared to nonirradiated (normal) corneas using TUNEL and cell viability/cytotoxicity assays. Corneal sections underwent TUNEL to identify DNA strand breaks generated by apoptosis. Apoptosis is a relatively rare occurrence in normal corneas as evidenced by the dearth of TUNEL⁺ cells in naïve corneal tissue (Fig. 1A). However, the TUNEL assay revealed widespread apoptosis in gamma-irradiated corneas, consistent with previous studies demonstrating that gamma irradiation can induce cellular apoptosis.²³ These findings were confirmed with a cell viability/cytotoxicity assay that allowed for the detection of live and dead cells. Live cells with intracellular esterase activity were stained with calcein AM, while dead cells with disrupted cell membranes were stained with ethidium homodimer-1. We found that most of the cells in the nonirradiated corneas were viable (data not shown). However, in gamma-irradiated corneas, viable cells were absent universally and all cells were positive for ethidium homodimer-1, indicating widespread cell death (Fig. 1B).

Full-Thickness Irradiated Corneal Allografts Opacified Rapidly

Penetrating keratoplasty was performed using irradiated C57BL/6 allografts, nonirradiated C57BL/6 allografts, and BALB/c recipients ($n = 6$ mice/group). Irradiated corneas were remarkably clear in the immediate postoperative period (Figs. 2A, 2B). However, irradiated allograft opacity rose between postoperative days 2 and 14, and average opacity exceeded 4+ by day 10. These findings differed significantly from nonirradiated allografts, which peaked in opacity on day 5 and cleared between days 5 and 14. Irradiated allograft opacity was significantly elevated by day 10 compared to nonirradiated allograft opacity ($P < 0.001$). All irradiated allografts (100%) failed by day 10, yielding a median survival time (MST) of 10 days (Fig. 3A). In contrast, 66.7% of nonirradiated allografts survived beyond day 21, and 50% survived beyond day 56 for an MST of 42 days. The survival of nonirradiated allografts was superior to the survival of irradiated allografts ($P < 0.001$).

Full-Thickness Irradiated Corneal Allografts Opacified Despite Immunosuppression

The most common cause of corneal allograft failure is immune rejection. Therefore, we evaluated the effect of immunosuppression on irradiated allograft survival. Irradiated allograft recipients received topical or topical plus systemic dexamethasone to determine the effect of immunosuppression on irradiated allograft survival ($n = 6$ mice/group). Immunosuppression did not improve irradiated allograft survival, as all immunosuppressed irradiated allografts (100%) failed by day 10, yielding an MST of 10 days (Fig. 3B).

Full-Thickness Irradiated Corneal Isografts Also Opacified Rapidly

Syngeneic corneal transplantation was performed to investigate further the possibility of immune rejection being the cause of irradiated graft failure. Syngeneic corneal transplantation was performed using irradiated C57BL/6 isografts, nonirradiated C57BL/6 isografts, and C57BL/6 recipients ($n = 6$ mice/group). The survival of irradiated isografts closely mirrored the survival of irradiated allografts, with all irradiated isografts (100%) failing by day 10 for an MST of 10 days (Fig. 3C). All nonirradiated isografts (100%) survived beyond postoperative day 56. The difference between nonirradiated isograft survival and irradiated isograft survival was statistically significant ($P < 0.001$).

T-Cells from Irradiated Allograft Recipients Did Not Exhibit a Secondary Alloimmune Response

MLR was used to provide an *in vitro* assessment of T-cell proliferation in response to the presentation of donor alloantigens ($n = 3$ mice/group per trial). T-cells from naïve BALB/c mice with no previous exposure to donor alloantigens were used as the negative control, while T-cells from BALB/c mice with actively rejecting nonirradiated corneal allografts were used as the positive control. BALB/c mice that received irradiated allografts were either left untreated or treated with a combination of topical and systemic dexamethasone. T-cell proliferation in response to alloantigen presentation was minimal for the naïve, irradiated allograft, and dexamethasone-treated irradiated allograft groups (Fig. 4A). In contrast, T-cell proliferation was robust for the actively rejecting corneal allograft group, indicating alloantigen exposure and sensitization. The difference in T-cell proliferation was significant when comparing the naïve, irradiated allograft, and immunosuppressed irradiated allograft groups with the actively rejecting corneal allograft group ($P < 0.001$).

Gamma-Irradiated Allografts Did Not Elicit an Alloreactive DTH Response

The DTH assay was used to provide an *in vivo* assessment of the immune response to donor alloantigens ($n = 4$ –6 mice/group). Naïve BALB/c mice with no previous exposure to donor alloantigens were used as a negative control, while BALB/c mice with actively rejecting nonirradiated corneal allografts were used as a positive control. Specific ear swelling was 3.42 ± 1.32 μ m for the naïve group, 9.83 ± 3.40 μ m for the actively rejecting nonirradiated allograft group, 3.08 ± 1.00 μ m for the irradiated allograft group, and 3.33 ± 0.92 μ m for the dexamethasone-treated irradiated allograft group (Fig. 4B). Differences in specific ear swelling were significant when comparing the naïve, irradiated allograft, and immunosuppressed irradiated allograft groups to the actively rejecting allograft group ($P \leq 0.01$).

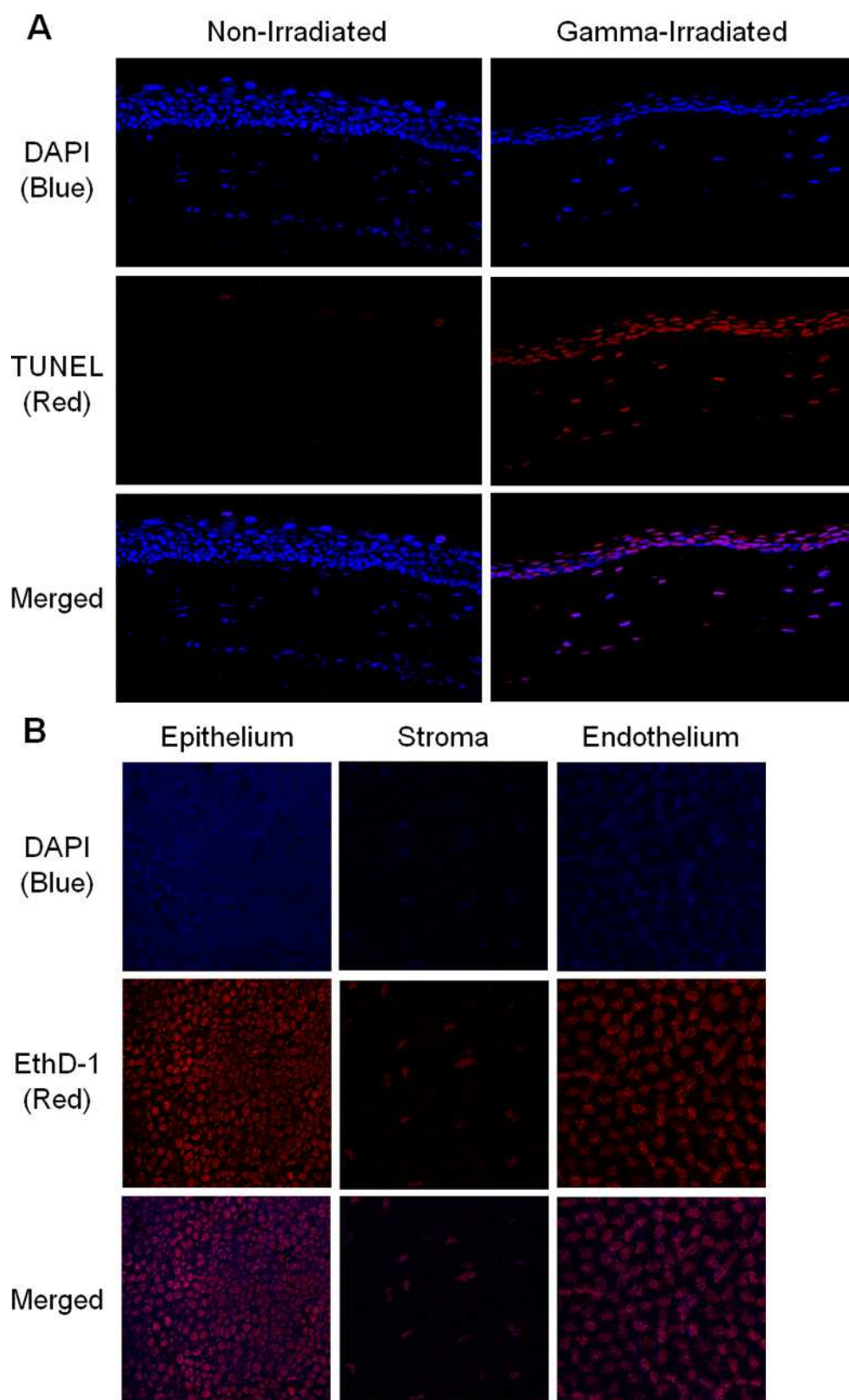


FIGURE 1. Gamma irradiation devitalized the donor corneal cells. **(A)** Representative TUNEL staining of nonirradiated and irradiated corneal sections indicating widespread cellular apoptosis in irradiated corneas (DAPI = *blue*, TUNEL = *red*). **(B)** Representative cell viability/cytotoxicity staining of irradiated corneal epithelium, stroma, and endothelium. All of the irradiated corneal cells were positive for ethidium homodimer-1 (EthD-1), indicating widespread plasma membrane disruption and cell death (DAPI = *blue*, EthD-1 = *red*).

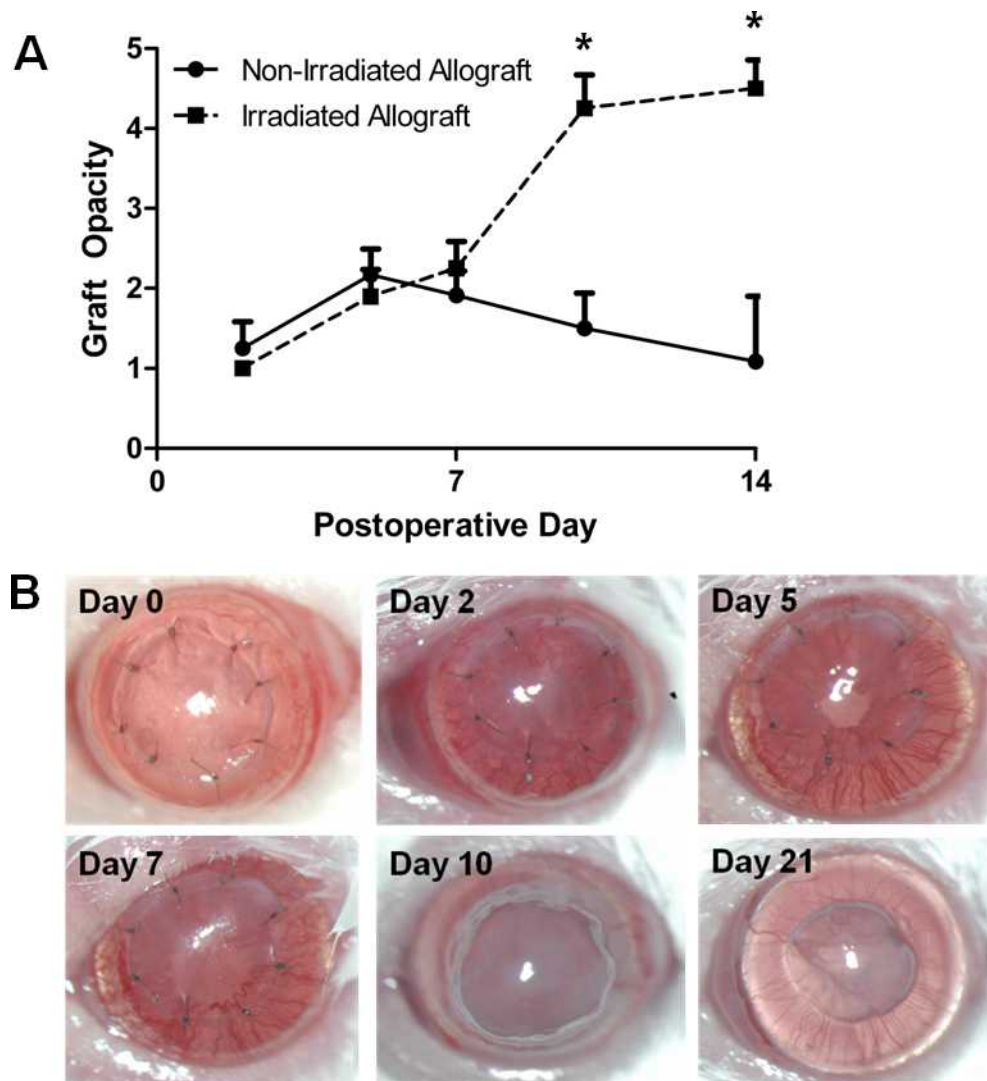


FIGURE 2. Gamma-irradiated corneal allograft opacity. **(A)** Nonirradiated allograft opacity peaked at day 5 before trending downwards, whereas irradiated allograft opacity rose between postoperative days 2 and 14 ($*P < 0.001$). **(B)** Representative irradiated allograft opacity. *Day 0*: minimal-to-moderate corneal opacity was evident throughout the penetrating keratoplasty procedure. *Day 2*: epithelial opacity (1^+) was present following tarsorrhaphy removal. *Day 5*: stromal opacity ($1-2^+$) began to develop. *Day 7*: stromal opacity ($2-3^+$) became increasingly evident. *Day 10*: significant opacity (5^+) and graft protrusion evident following corneal suture removal. *Day 21*: significant opacity (4^+) remained throughout the observation period.

T-Helper (Th) Cell Infiltration of Irradiated Allografts Was Negligible

Th cell infiltration of corneal grafts has been described in corneal allograft rejection.²⁴ Real-time PCR was used to determine the corneal mRNA expression of Th1-associated interferon (IFN)- γ , Th17-associated interleukin (IL)-17A, and Th2-associated IL-4 ($n = 3$ corneas/group per trial). The mRNA levels of IFN- γ , IL-17A, and IL-4 were elevated significantly in rejected allografts compared to naïve corneas, irradiated allografts, and immunosuppressed irradiated allografts ($*P \leq 0.001$, Fig. 5). The expression of IFN- γ and IL-17A was minimal, and IL-4 was undetectable in the corneas of irradiated allograft recipients.

DISCUSSION

T-cells are required for the rejection of corneal allografts.^{5,6} Rejected corneal allografts contain a mixed cellular infiltrate

that includes CD4⁺ (helper) and CD8⁺ (cytotoxic) T-cells.²⁴ Donor-specific CD4⁺ T-cells are thought to damage corneal allografts by secreting inflammatory mediators, activating ancillary effector cells, and inducing cellular apoptosis.²⁵ Abrogating the CD4⁺ T-cell response dramatically increases the rate of corneal allograft survival.²⁶⁻²⁹ Several CD4⁺ T-cell subsets have been implicated in the immunopathogenesis of corneal allograft rejection including Th1, Th2, and Th17 cells. Th1 cells mediate corneal allograft rejection under normal conditions by secreting proinflammatory cytokines (e.g., IFN- γ) and activating auxiliary effector cells (e.g., macrophages).^{25,30,31} IL-4, IL-5, and IL-13-secreting Th2 cells contribute to corneal allograft rejection in cases of allergic disease, such as airway hyperreactivity or allergic conjunctivitis.^{32,33} The role of Th17 cells in corneal allograft rejection remains unclear as IL-17 has been implicated in early transplant rejection and ocular immune privilege.^{34,35} In the absence of CD4⁺ T-cells, CD8⁺ T-cells can mediate corneal allograft rejection independently.³⁶ Regardless of the exact role of each T-cell subset, Th

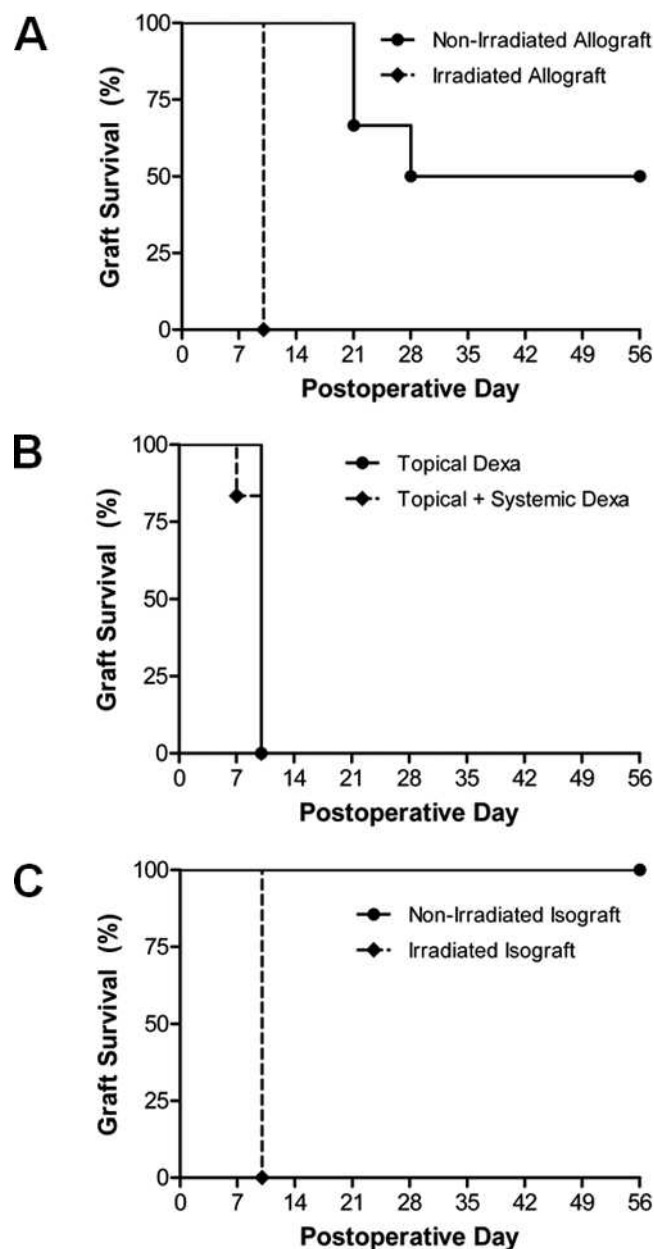


FIGURE 3. Gamma-irradiated corneal graft survival. (A) Survival curve demonstrating that no irradiated allografts survived beyond day 10, whereas 50% of nonirradiated allografts ($n = 6$ mice/group) survived to day 56 ($P < 0.001$). (B) Survival curve demonstrating that neither topical dexamethasone treated nor combined topical and systemic dexamethasone treated irradiated allografts ($n = 4-6$ mice/group) survived beyond postoperative day 10. *Dexa*: dexamethasone treated. (C) Survival curve demonstrating that no irradiated isografts survived beyond day 10, whereas 100% of nonirradiated isografts ($n = 6$ mice/group) survived to day 56 ($P < 0.001$).

cells are central to the immunopathogenesis of corneal allograft rejection.

Normal corneal architecture and viable corneal cells are necessary for the maintenance of corneal clarity.³⁷ Following gamma irradiation, human corneas have been shown to exhibit altered cellular architecture consistent with devitalized epithelial cells and keratocytes.³⁸ TUNEL and cell viability/cytotoxicity assays were performed to further characterize the devitalization of corneal cells induced by gamma irradiation. The TUNEL assay demonstrated widespread corneal cell

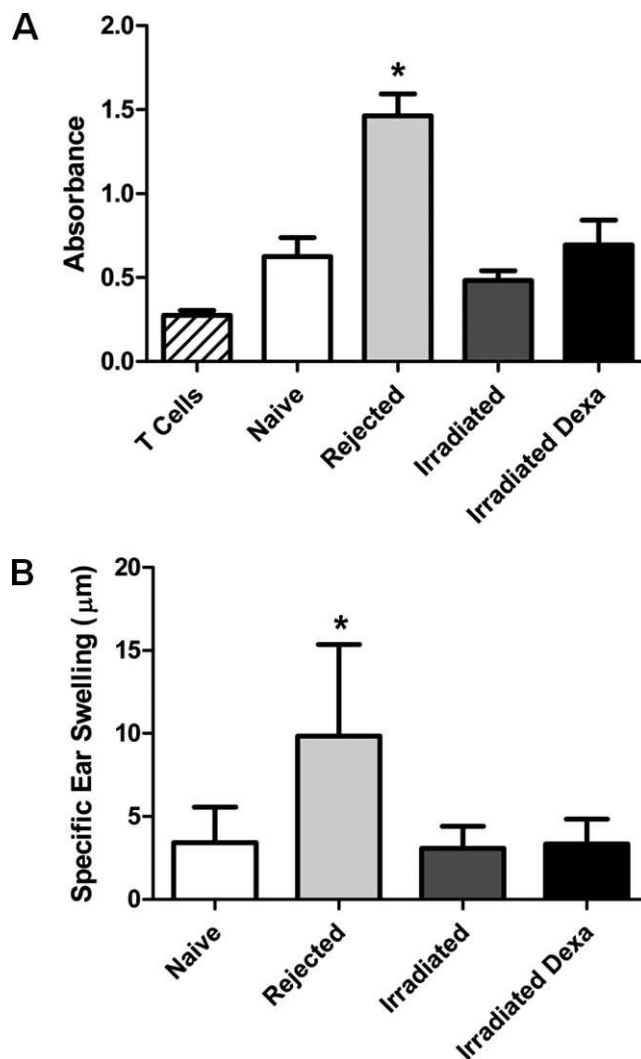


FIGURE 4. Gamma irradiation reduced corneal allogenicity. (A) Mixed lymphocyte reaction results demonstrating that T-cells from irradiated allograft recipients ($n = 3$ mice/group) do not exhibit a secondary alloimmune response (representative results from two independent trials, $*P < 0.001$). (B) Delayed-type hypersensitivity reaction demonstrating that irradiated corneas ($n = 4-6$ mice/group) do not elicit an alloreactive response in vivo ($*P \leq 0.01$). *Dexa*: topical and systemic dexamethasone treated.

apoptosis, while the cell viability/cytotoxicity assay demonstrated widespread corneal cell death. Despite the devitalization of donor corneal cells, irradiated allografts were remarkably clear in the immediate postoperative period; however, all irradiated allografts failed by postoperative day 10. Corticosteroids are the mainstay of treatment for corneal allograft rejection, but neither topical nor combined topical and systemic corticosteroid treatment improved irradiated allograft survival. Furthermore, syngeneic corneal transplantation demonstrated that irradiated isografts failed at the same rate as irradiated allografts. In contrast, nonirradiated allografts enjoyed a 50% survival rate, and nonirradiated isografts enjoyed a 100% survival rate. Although immune rejection is the leading cause of corneal transplantation failure, grafts can fail for a variety of reasons, including endothelial cell failure.³⁹ These findings suggest that the inability of irradiated corneal grafts to remain optically clear was not immune-mediated.

Cell preservation techniques that devitalize cells (e.g., cryopreservation) have demonstrated immunosuppressive

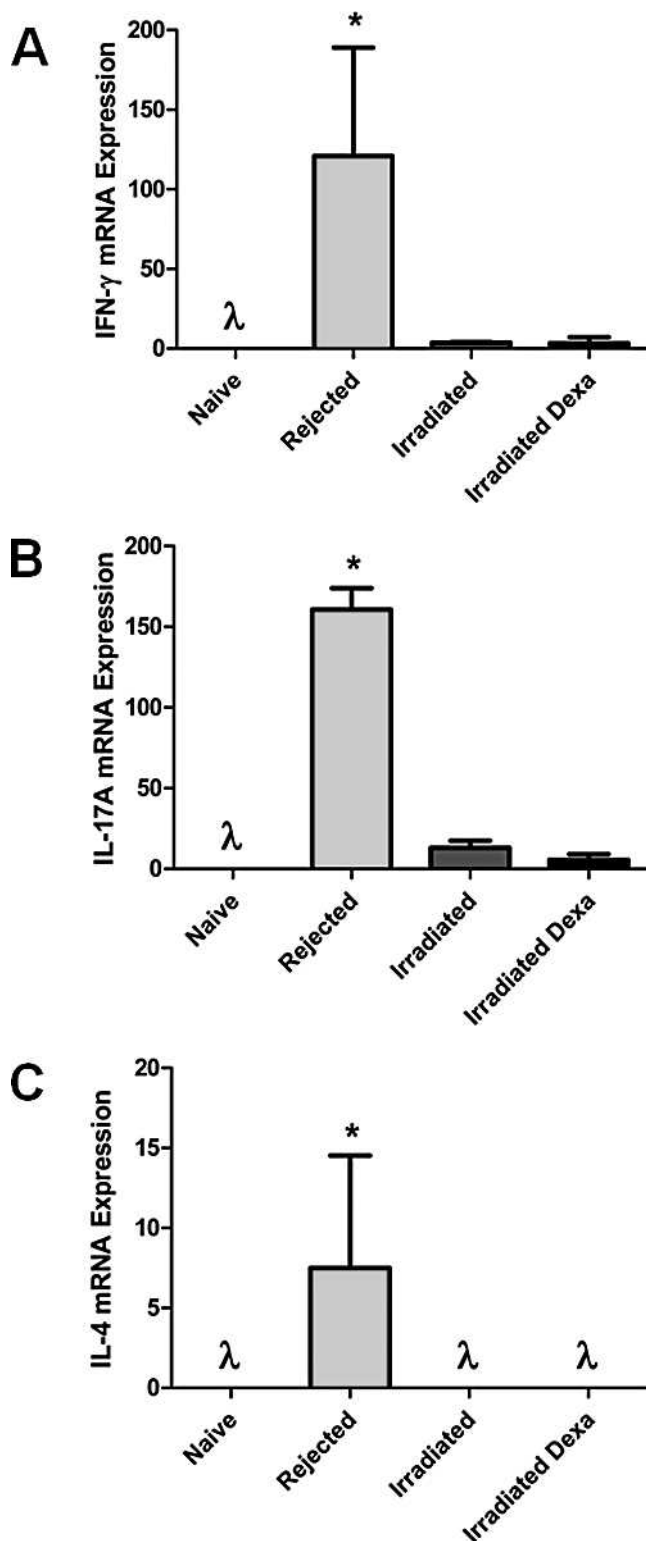


FIGURE 5. Gamma irradiation reduced T-helper cell-associated cytokine expression. Real-time PCR results demonstrating that (A) Th1-associated IFN- γ , (B) Th17-specific IL-17A, and (C) Th2-associated IL-4 were elevated significantly in rejected nonirradiated allografts compared to naive corneas and irradiated allografts. Representative results from two independent trials ($n = 3$ transplanted corneas/group, $*P \leq 0.001$, λ indicates lack of detection). Dexa: topical and systemic dexamethasone treated.

effects in a variety of experimental models.^{40,41} High-dose gamma irradiation has been shown to reduce the risk of immune rejection in experimental models of tracheal and islet cell transplantation.^{42,43} Because T-cells are required for the rejection of corneal allografts, MLR and DTH assays were performed to evaluate T-cell alloreactivity. The MLR assay demonstrated that T-cells isolated from irradiated allograft recipients did not exhibit a secondary alloimmune response. The DTH assay demonstrated that irradiated allografts did not elicit an alloreactive DTH response. Furthermore, the reduced or absent corneal expression of Th1-associated IFN- γ , Th17-associated IL-17A, and Th2-associated IL-4 strongly suggests that gamma irradiation decreased or eliminated the corneal infiltration of potentially pathogenic Th cells. The low-level infiltration of IFN- γ and IL-17A mRNA-expressing cells may have been the result of non-specific inflammation. The reduced allogenicity of gamma-irradiated corneas is likely related to the devitalization of potentially antigenic corneal cells, including resident antigen-presenting cells. Although extending the duration of corneal allograft storage has been shown to decrease the number of passenger leukocytes, this does not necessarily increase corneal allograft survival.⁴⁴ Given that the alloantigens that stimulate alloreactivity are primarily cell-associated, devitalizing the donor corneal cells could dramatically decrease the graft's alloantigen load. These findings also may apply to other biocompatible acellular corneal grafts, for example decellularized corneal tissue or acellular collagen scaffolds. In fact, a prospective, randomized clinical trial investigating the use of glycerol-cryopreserved corneal tissue in anterior lamellar keratoplasty reported no cases of allorejection.⁴⁵ Although isolated anterior grafts do not elicit endothelial immune reactions, epithelial rejection and stromal rejection remain tangible concerns.⁴⁵

VisionGraft Sterile Corneas have been used in a variety of clinical procedures, including anterior lamellar keratoplasty, tectonic keratoplasty, glaucoma patch grafting, and keratoprosthesis implantation.^{15,16} To be chosen for gamma irradiation, a cornea must exhibit healthy, clear stroma and comply with the Eye Bank Association of America's standards for screening and procurement.⁴⁶ The adaptation of VisionGraft Sterile Corneas promises to increase the supply and extend the shelf-life of donated corneas. Taken together, our results indicate that gamma-irradiated corneas do not elicit T-cell-mediated alloimmunity. This is significant given the importance of T-cells in the rejection of nonirradiated corneal allografts. Although our experimental results suggest that gamma-irradiated corneas may not be suitable for use in penetrating keratoplasty, their reduced allogenicity potentially supports their use in procedures such as anterior lamellar keratoplasty or keratoprosthesis implantation.

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